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SYNERGY IN SEQUENTIAL INACTIVATION OF *CRYPTOSPORIDIUM PARVUM* WITH OZONE/FREE CHLORINE AND OZONE/MONOCHLORAMINE

JASON L. RENNECKER[M], AMY M. DRIEDGER[M], SARA A. RUBIN and BENITO J. MARIÑAS*[M]

Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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Abstract—The main objective of this study was to investigate the inactivation kinetics of *Cryptosporidium parvum* oocysts with sequential disinfection schemes involving ozone as a primary disinfectant, and free chlorine or monochloramine as a secondary disinfectant. Two types of synergistic effects were observed. Ozone pre-treatment resulted in the removal of the relatively more pronounced initial lag phases observed for monochloramine and hypochlorous acid. An additional and more important synergistic effect was an enhancement in the rate of secondary inactivation with both hypochlorous acid and monochloramine after complete removal of the lag phase by ozone pre-treatment. A stronger synergy was observed at a lower temperature. The secondary inactivation rate was 1.1–2.8 (hypochlorous acid) and 2.4–9.2 (monochloramine) times faster than the corresponding post lag-phase primary inactivation rate at respective temperatures of 30–10°C. Consistency between the two viability assessment methods, modified *in-vitro* excystation and animal infectivity, was demonstrated or shown for both primary inactivation with ozone and secondary inactivation with ozone/monochloramine.

Key words—animal infectivity, *Cryptosporidium parvum*, free chlorine, inactivation kinetics, *in-vitro* excystation, monochloramine, ozone, synergy, temperature dependence

INTRODUCTION

Most drinking water disinfection systems in the United States treating surface water were originally designed, or subsequently modified, to control microbial contaminants regulated under the Surface Water Treatment Rule (SWTR) (US EPA, 1989), i.e., enteric viruses and *Giardia lamblia* cysts. Unfortunately, the *CT* (product of average disinfectant concentration and contact time) parameters achieved in these systems are generally inadequate for inactivating the emerging protozoan parasite *Cryptosporidium parvum*. High *CT* requirements for both free and combined chlorine (Korich *et al.*, 1990; Gyürék *et al.*, 1997; Oppenheimer *et al.*, 1997) make these two common disinfectants practically ineffective for inactivating *C. parvum* oocysts in drinking water. In contrast, ozone and chlorine dioxide are more effective in controlling *C. parvum* oocysts (Korich *et al.*, 1990; Finch *et al.*, 1993a; Rennecker *et al.*, 1999a; Ruffell *et al.*, 2000). However, many existing ozone and chlorine dioxide disinfection systems will need upgrading in order to provide the high *CT* requirements anticipated for *C. parvum* oocysts.

Ozone and chlorine dioxide are both strong oxidizing agents that, in addition to reacting with pathogens, decompose by reacting with other common constituents of natural waters. As a result, treatment plants using ozone or chlorine dioxide as "primary" disinfectant also apply a secondary disinfectant, free or combined chlorine, in order to provide a residual in the distribution system as required by the Disinfectants/Disinfection By-Products Rule (US EPA, 1998).

Sequential inactivation of *C. parvum* oocysts with ozone or chlorine dioxide as primary disinfectants and free or combined chlorine as secondary disinfectants is currently under investigation (Finch *et al.*, 1998; Gyürék *et al.*, 1996; Liyanage *et al.*, 1997; Oppenheimer *et al.*, 1997; Rennecker *et al.*, 1999b; Driedger *et al.*, 2000). Despite these efforts, no agreement has been reached about the efficiency of these processes. Reasons for this impasse are the variability of the scarce data available, and the high

*Author to whom all correspondence should be addressed. Tel.: +1-217-333-6961; fax: +1-217-333-6968; e-mail: marinas@uiuc.edu

Table 1. Summary of single-step and sequential disinfection tests and experimental conditions

		Time elapsed from oocyst shedding (days)			T (°C)	Control		Ozone (pH 7)			Free chlorine (pH 6)		Monochloramine (pH 8)	
Test	Lot	Shipment	Cleaning	Testing		S_c/EO_c	$(N/N_0)_c$	C (mg/l)	T (min)	N/N_0	C (mg/l)	T (min)	C (mg/l)	T (min)
O-1	A	7	9	10	20	1.57	0.900	0.48	0.5–6.3	–	–	–	–	–
O-2	B	9	10	11	20	1.72	0.815	0.48	1–12	–	–	–	–	–
O-3	C	11	13	16	20	2.11	0.525	0.49	0.5–13	–	–	–	–	–
O-4	C	11	13	46	4	2.28	0.450	1.04	1.9–34	–	–	–	–	–
O-5	C	11	13	87	10	1.50	0.498	0.61	0.8–18	–	–	–	–	–
O-6	C	11	13	115	30	0.96	0.487	0.35	0.6–2.1	–	–	–	–	–
C-1	A	7	9–10	11	20	2.54	0.687	–	–	–	7.9	33–387	–	–
C-2	A	7	9–10	20	30	2.79	0.671	–	–	–	8.2	16–144	–	–
C-3	A	7	9–10	26	4	2.64	0.803	–	–	–	8.1	221–2000	–	–
C-4	A	7	9–10	52	10	2.87	0.744	–	–	–	7.9	111–888	–	–
M-1	A	7	9–10	39	20	2.78	0.793	–	–	–	–	–	8.0	177–1400
M-2	A	7	9–10	114	30	3.73	0.641	–	–	–	–	–	7.6	83–576
M-3	A	7	9–10	124	10	3.84	0.385	–	–	–	–	–	7.8	440–3360
M-4	A	7	9–10	159	4	2.72	0.248	–	–	–	–	–	7.4	1090–6170
OC-1	A	7	9	31	20	1.72	0.653	0.49	0.71	0.591	7.9	28–256	–	–
OC-2	A	7	9	14	20	1.91	0.854	0.48	1.45	0.690	8.0	10–90	–	–
OC-3	A	7	9	26	20	1.60	0.657	0.44	3.17	0.207	8.2	9–89	–	–
OC-4	C	11	13	38	20	2.05	0.488	0.48	2.90	0.141	8.5	10–85	–	–
OC-5	C	11	13	41	30	2.11	0.594	0.37	1.09	0.061	7.6	8–64	–	–
OC-6	C	11	13	100	10	1.98	0.350	0.62	5.68	0.167	7.7	27–219	–	–
OC-7	C	11	13	112	4	1.76	0.324	0.74	13.5	0.131	8.2	20–81	–	–
OM-1	B	9	10	42	20	1.61	0.753	0.45	0.77	0.874	–	–	8.1	156–1220
OM-2	A	7	9	47	20	1.37	0.616	0.49	1.45	0.634	–	–	8.0	25–225
OM-3	B	9	10	37	20	1.40	0.758	0.49	2.86	0.431	–	–	8.0	35–281
OM-4	C	11	13	80	30	2.09	0.537	0.34	0.88	0.234	–	–	8.2	9–73
OM-5	C	11	13	91	20	2.27	0.440	0.47	3.00	0.150	–	–	8.2	23–185
OM-6	C	11	13	103	10	1.90	0.354	0.60	5.84	0.182	–	–	8.2	61–491
OM-7	C	11	13	110	4	2.09	0.462	0.71	14.1	0.160	–	–	8.5	45–182
OM-8	D	15	36	39	4	2.37	0.808	0.73	13.6	0.333	–	–	7.7	21–169
OM-9	D	15	36	78	20	1.50	0.814	0.45	3.13	0.223	–	–	8.1	48–200

level of resources required to produce the necessary data by the viability assessment method used in most studies, i.e., animal infectivity. Recent data sets for primary disinfection with ozone (Rennecker *et al*., 1999a), and chlorine dioxide (Ruffell *et al*., 2000) for which oocyst viability was assessed by a modified *in-vitro* excystation method were shown to be consistent with animal infectivity results reported in the literature. The use of the modified *in-vitro* excystation method, simpler, faster and more accurate than current animal infectivity assays, might also allow the development of comparable data sets for sequential disinfection if a maximum overall inactivation efficiency of approximately 99.5% is deemed adequate.

The objectives of this study were to investigate the inactivation kinetics of *C. parvum* oocysts with sequential disinfection schemes involving ozone as primary disinfectant, and free chlorine or monochloramine as secondary disinfectant. Experiments were performed to assess the role of ozone pretreatment level and temperature in the sequential inactivation of *C. parvum* oocysts. An additional goal of this research was to provide additional evidence supporting the consistency between the modified *in-vitro* excystation and animal infectivity methods.

MATERIALS AND METHODS

Oocysts

Experiments were performed with bovine-source Iowa strain *C. parvum* oocysts obtained from the University of Arizona. Oocyst stocks were cleaned and stored following procedures described previously (Rennecker *et al*., 1999a; Ruffell *et al*., 2000; Driedger *et al*., 2000). Oocysts from four different shipments (lots A–D, Table 1) were used for the various experiments of the study. Times elapsed from oocyst shedding at the University of Arizona to shipment, cleaning, and testing for each experimental set are presented in Table 1.

Experimental matrix

Experiments were performed to assess the variability in the ozone inactivation kinetics obtained for different oocyst lots (tests O-1–3) at 20°C. The effect of temperature (4–30°C) on ozone inactivation kinetics was investigated with oocyst lot C (tests O-3–6). All ozone disinfection experiments were performed in a semi-batch reactor with the oocysts suspended in 0.01 M phosphate buffer solution (PBS) at pH 7. The dissolved ozone concentrations used ranged from 0.35 to 1.0 mg/l. Each ozone disinfection test was designed to obtain an inactivation curve with generally 8–9 data points plus control and blank samples.

Experiments were also performed to determine the effect of temperature (4–30°C) on the inactivation kinetics of *C. parvum* with free chlorine (tests C-1–4) and monochloramine (tests M-1–4), both used as primary disinfectants. These tests served as the baseline for secondary inactivation experiments with the same disinfectants. All experiments were performed in a batch reactor with 0.01 M PBS at pH 6 (free chlorine) or pH 8 (pre-formed monochloramine), and target dissolved disinfectant concentration of 8.0 mg/l as Cl_2. Each test was designed to obtain an inactivation curve with generally 8–9 data points and corresponding control and blank samples. The pH of 6 was selected for free chlorine because previous studies (Driedger *et al*., 2000) have shown that hypochlorous acid is the main free chlorine species responsible for inactivation of *C. parvum*. The pH of 8 was selected for monochloramine to minimize its decomposition.

Sequential disinfection experiments were performed by first treating oocysts with ozone at the levels indicated in Table 1, and subsequently exposing the pre-treated oocysts to free chlorine (tests OC-1–7) or monochloramine (tests OM-1–9). Ozone pre-treatment was performed in a semi-batch reactor with 0.01 M PBS at pH 7 and the same temperature of the secondary disinfection step. Experimental conditions for secondary inactivation with free chlorine and monochloramine were the same as those described for primary inactivation with these disinfectants. Sequential disinfection tests were designed to assess the role of both preozonation *CT* (tests OC-1–3, OM-1–3) and temperature (tests OC-4–7, OM-4–8). Test OM-9 was performed to compare secondary inactivation efficiencies assessed by animal infectivity and modified *in-vitro* excystation using split samples. Each sequential disinfection test was designed to obtain a secondary inactivation curve with generally eight data points plus control and blank samples.

Ozone disinfection

Ozone disinfection (tests O-1–6) experiments were performed in a semi-batch reactor apparatus. Experimental components used and methods followed were those described by Rennecker *et al*. (1999a) with the exception that a lower dose of approximately 7×10^5 oocysts was used for each experimental run.

Free and combined chlorine disinfection

Primary inactivation experiments with free and combined chlorine (tests C-1–4 and M-1–4) were performed with a batch reactor. Experimental components used and methods followed for the free chlorine experiments were the same as those described by Driedger *et al*. (2000). Monochloramine solutions were prepared by dissolving 3.054 g of NH_4Cl (Fisher Scientific, Itasca, IL) in 100 ml of 0.01 M PBS at pH 8. Two ml of this stock solution were added to approximately 750 ml of 0.01 M PBS (pH 8) contained in a 2 l volumetric flask. In a separate 1-l volumetric flask, a predetermined amount of 4–6% sodium hypochlorite solution (Fisher Scientific, Itasca, IL), corresponding to approximately 16 mg/l as Cl_2, was added to 1 l of 0.01 M PBS (pH 8) and subsequently stirred to ensure complete mixing. The sodium hypochlorite/buffer solution was added, under continuous mixing, to the 2-l flask containing the ammonium chloride solution. The 2-l flask was subsequently brought to volume with 0.01 M (pH 8) PBS and mixed for 20 min. The solution was then transferred to a 2.33-l amber glass bottle and the pH was adjusted to 8.00 ± 0.05, if necessary, by the dropwise addition of NaOH or HCl. The monochloramine concentration was then determined by Standard Method 4500-Cl F (APHA *et al*., 1992), as well as by direct UV spectrophotometry. A spectrophotometer, Model 1601 (Shimadzu Scientific Instruments, Columbia, MD), with wavelength set at 243 nm was used for the latter method. Results obtained by direct spectrophotometry using a molar absorptivity of 461 M^{-1} cm^{-1} (Kumar *et al*., 1986) were found to agree within 5% of those corresponding to Standard Method 4500-Cl F. The 2.33-l reactor containing the monochloramine solution was immersed in a water bath set at the target experimental temperature. Other experimental components used and methods followed for the monochloramine experiments were the same as those described for free chlorine by Rennecker *et al*. (1999b) and Driedger *et al*. (2000).

Sequential disinfectant inactivation experiments

Primary and secondary inactivation of *C. parvum* oocysts with ozone/free chlorine (tests OC-1–7) or ozone/monochloramine (tests and OM-1–9) was investigated in a manner similar to that described previously for the single-step disinfection experiments. Additional steps specific to the two-step inactivation procedure were described by Rennecker *et al.* (1999b) and Driedger *et al.* (2000).

Viability assessment

Oocyst viability of control and disinfected samples was assessed by the modified *in-vitro* excystation assay developed by Rennecker *et al.* (1999a) for all experimental sets presented in Table 1. The various steps comprising this method have been described in previous publications (Rennecker *et al.*, 1999a; Ruffell *et al.*, 2000). Oocyst viability (N/N_0) was calculated with the expression (Rennecker *et al.*, 1999a):

$$\frac{N}{N_0} = \frac{\mathrm{S/X}}{\mathrm{IO} + \mathrm{EO}} \tag{1}$$

where IO, EO and S are the numbers of intact oocysts, excysted oocysts, and sporozoites, respectively, counted by phase contrast microscopy at a magnification of 1000×. X is the ratio of sporozoites to excysted oocysts in the control, S_c/EO_c, given in Table 1 for each test. The total number of entities, i.e., IO + EO + S, enumerated for each sample was at least 800 so that inactivation efficiencies as high as 99.5% could be determined with coefficient of variation within 50%. The number of sporozoites and excysted oocysts observed in control and treated samples were corrected to account for their presence in blank (i.e., oocyst stock) samples.

The validity of the modified *in-vitro* excystation method to represent inactivation was checked with an ozone/monochloramine experiment (test OM-9). The experimental methods followed were the same as those used for other sequential experiments of this study with the exception of doubling the oocyst dose per run (1.45×10^7 oocysts). The resulting control and disinfected samples were split for viability assessment by both modified *in-vitro* excystation and animal infectivity methods. The animal infectivity samples were shipped by overnight delivery to the University of Arizona for viability assessment according to the following protocol. Four- to six-day old neonatal CD-1 out-bred mice were administered doses of oocysts prepared in sterile water adjusted to pH 7. Doses were prepared by dilution of samples after sample density determination with a hemacytometer, and based on inactivation efficiencies obtained by the modified *in-vitro* excystation assay. The oocysts were administered orally in 10-μl volumes. A total of 16–24 mice were inoculated per sample. The animals were sacrificed seven days post inoculation and a 2-cm section of the terminal ileum was removed. The tissue samples were fixed in formalin, embedded in paraffin, sectioned, mounted on slides, stained, and examined for signs of infection. Samples were labeled as either positive or negative for infection. Oocyst viability was determined with the following dose–response formula (Finch *et al.*, 1993b):

$$\ln\left(\frac{P}{1-P}\right) = -a_1 + a_2 \log_{10}(\mathrm{VO}) \tag{2}$$

in which VO is the number of viable oocysts in the inoculum, and P is the fraction of mice infected. The parameters a_1 and a_2 are empirical constants obtained by fitting experimental dose–response data with equation (2).

RESULTS AND DISCUSSION

Primary disinfection with ozone

The inactivation kinetics of *C. parvum* oocysts with ozone at pH 7 and 20°C for three of the oocyst lots used in this study are presented in Fig. 1 (tests O-1–3, Table 1). Consistent with previous observations with ozone (Rennecker *et al.*, 1999a), each inactivation curve was characterized by a relatively short lag phase with little inactivation followed by pseudo-first-order inactivation kinetics. The viability of the oocyst stock was less than 100% for all three lots with lot C having the lowest viability (51%).

The inactivation curves were fitted with the delayed Chick–Watson model (Rennecker *et al.*, 1999a):

$$\frac{N}{N_0} = \begin{cases} \left(\dfrac{N}{N_0}\right)_c \\ \text{if } CT \le CT_{\mathrm{lag}} = \dfrac{1}{k}\ln\left\{\left(\dfrac{N_1}{N_0}\right)\left(\dfrac{N_0}{N}\right)_c\right\} \\ \dfrac{N_1}{N_0}\exp(-kCT) = \exp(-k\{CT - CT_{\mathrm{lag}}\}) \\ \text{if } CT > CT_{\mathrm{lag}} = \dfrac{1}{k}\ln\left\{\left(\dfrac{N_1}{N_0}\right)\left(\dfrac{N_0}{N}\right)_c\right\} \end{cases} \tag{3}$$

where k is the post-shoulder second-order inactivation rate constant in l/(mg × min), C is the dissolved ozone concentration in mg/l, T is the contact

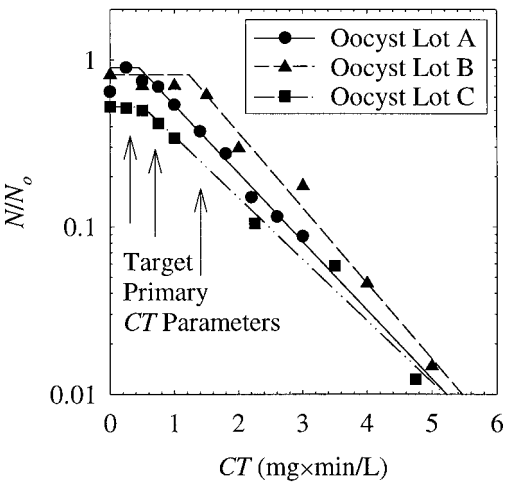

Fig. 1. Kinetics of primary inactivation of *C. parvum* oocysts (lots A, B, and C) with ozone at 20°C and pH 7. Arrows indicate target pre-treatment *CT* levels prior to secondary disinfection with free chlorine (pH 6) or monochloramine (pH 8).

time in min, N_1/N_0 is the intercept with the ordinate axis resulting from extrapolation of the pseudo-first-order line, $(N/N_0)_c$ is the viability of the control (Table 1), and CT_{lag} is the lag phase CT. The lines presented in Fig. 1 resulted from fitting the data with equation (3). The resulting N_1/N_0 values were 1.4, 2.9, and 0.81 for lots A, B, and C, respectively. The respective rate constants were 0.942, 1.04, and 0.845 l/(mg × min) which were within 18% of that of 1.02 l/(mg × min) reported by Rennecker *et al.* (1999a) for rodent-source Iowa strain oocysts.

The effect of temperature on the rate of *C. parvum* oocyst inactivation with ozone is shown in Fig. 2. Consistent with the observation by Rennecker *et al.* (1999a), the lag phase increased and the pseudo-first-order rate decreased with decreasing temperature within the range of 4–30°C investigated. Notice that although the temperature experiments were performed at times ranging from 16 days (20°C) to 115 days (30°C) after oocyst shedding, no significant change was observed in the viability of the control (Table 1) which averaged $(N/N_0)_c = 0.49$. Rate constants obtained by regression of each data set in Fig. 2 with equation (3) are plotted in Fig. 3 according to the Arrhenius expression:

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (4)$$

where A is the frequency factor in l/(mg × min), E_a is the apparent activation energy in J/mole, R = 8.314 J/(mole × K) is the ideal gas constant, and T is absolute temperature in K. The intercept with the ordinate axis resulting from extrapolating each inactivation curve was approximately $(N_1/N_0) = 0.81$, independently of temperature. Also shown in Fig. 3 is the line reported by Rennecker *et al.* (1999a) for the ozone inactivation of rodent-source Iowa strain oocysts ($A = 3.00 \times 10^{14}$ l/(mg × min), $E_a = 81{,}200$ J/mole). As depicted in Fig. 3, experimental k values obtained in this study were consistent (within 30%) with the temperature dependence by Rennecker *et al.* (1999a) with exception of that at 30°C that was 2.25 times higher than expected. The role of oocyst age in the occurrence of this discrepancy will be addressed in a subsequent section when discussing similar effects also observed for sequential inactivation.

Primary disinfection with free chlorine and monochloramine

The inactivation kinetics of *C. parvum* oocysts with free chlorine at pH 6 (approximately 96% of the free chlorine present as hypochlorous acid), and pre-formed monochloramine (pH 8) at temperatures of 4–30°C are presented in Figs 4 and 5, respectively. Similar to the observation for ozone, each inactivation curve was comprised of a lag phase followed by a pseudo-first-order inactivation rate. Notice that the lag phases obtained with free chlorine and monochloramine were somewhat more pronounced than those observed for ozone.

The primary inactivation experiments with free and combined chlorine were all performed with the same shipment of oocysts (lot A, Table 1). No trends were observed in the excystation efficiency of the free chlorine control, averaging $(N/N_0)_c = 0.74$,

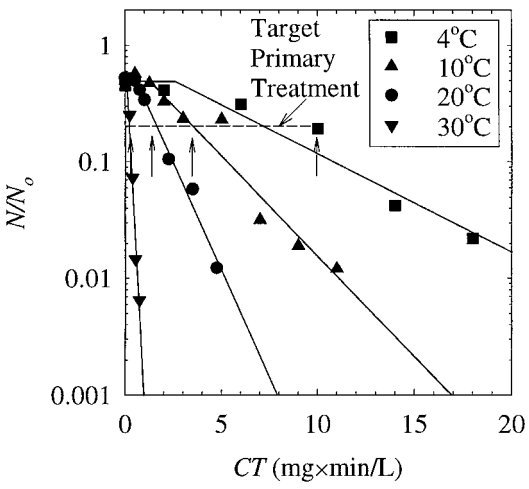

Fig. 2. Effect of temperature on the kinetics of primary inactivation of *C. parvum* oocysts (lots C) with ozone at pH 7. Arrows indicate target pre-treatment CT levels prior to secondary disinfection with free chlorine (pH 6) or monochloramine (pH 8).

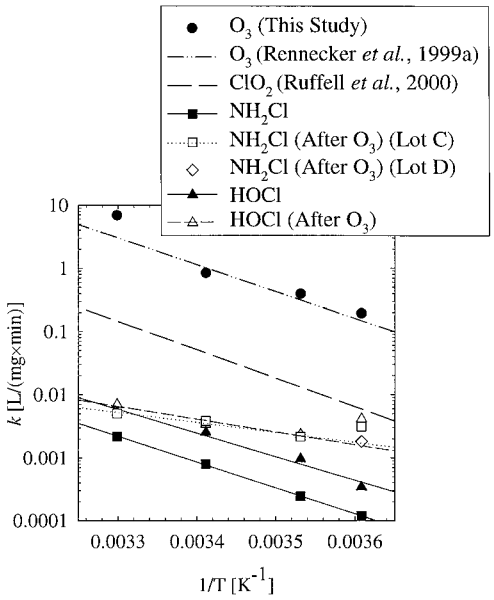

Fig. 3. Arrhenius plot of second-order rate constants for primary inactivation with ozone (pH 7, this study and Rennecker *et al.*, 1999a), chlorine dioxide (Ruffell *et al.*, 2000), free chlorine (pH 6) and monochloramine (pH 8), and for secondary inactivation with free chlorine (pH 6) and monochloramine (pH 8) after ozone pre-treatment (pH 7).

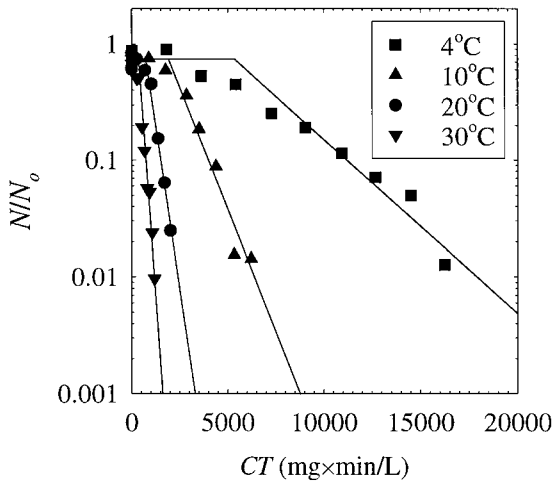

Fig. 4. Effect of temperature on the kinetics of primary inactivation of *C. parvum* oocysts (lots A) with free chlorine at pH 6.

with oocyst age. In contrast a decrease in $(N/N_0)_c$ was observed for monochloramine. The minimum excystation efficiency of the control, $(N/N_0)_c = 0.25$, was reached for the 4°C test performed with the oldest oocysts. It appeared that the oocysts were stable for approximately 3–4 months and deteriorated with subsequent storage time at 4°C.

Each inactivation curve in Figs 4 and 5 was fitted with equation (3). The resulting extrapolated intercept for free chlorine was $N_1/N_0 = 4.6$ independently of oocyst age. In contrast values for monochloramine dropped from $N_1/N_0 = 61$ at 20°C to $N_1/N_0 = 1.4$ at 4°C, when the respective oocysts used were 39 to 159 days old. The fitted rate constants are plotted in Fig. 3. The temperature dependence of the rate constants was generally consistent with equation (4) for both free chlorine and monochloramine as depicted in the figure. The parameters obtained by fitting the k values to equation (4) were $A = 1.29 \times 10^{10}$ l/(mg × min) and $E_a = 71{,}610$ J/mole for free chlorine, and $A = 7.93 \times 10^{10}$ l/(mg × min) and $E_a = 78{,}650$ J/mole for monochloramine. Notice that the apparent activation energies for free chlorine and monochloramine were within 12 and 3%, respectively, of the value $E_a = 81{,}200$ J/mole for ozone. Interestingly, the activation energy for chlorine dioxide, $E_a = 86{,}300$ J/mole (Ruffell *et al.*, 2000) was also within 7% of that for ozone (see Fig. 3), thus suggesting that there might be some similarities in the inactivation mechanisms of *C. parvum* oocysts by these four disinfectants.

Sequential disinfection with ozone/free chlorine and ozone/monochloramine

Effect of ozone pre-treatment CT. Results for the experiments designed to assess the effect of ozone pre-treatment CT on the secondary inactivation kinetics of free chlorine at pH 6 (tests OC-1–4) and monochloramine at pH 8 (tests OM-1–3, 5), and 20°C are presented in Figs 6 and 7, respectively. Filled and open symbols were used to represent primary and secondary inactivation curves, respectively. Filled symbols were also used to represent the control (without ozone pre-treatment) corresponding to each secondary inactivation curve. CT levels investigated for ozone pre-treatment, 0.35, 0.70 and 1.4 mg × min/l, are indicated with arrows in Fig. 1. These pre-treatment CT levels were chosen to be approximately 0.5, 1.0 and 2.0 times the observed CT_{lag}. The results obtained for secondary disinfection with free chlorine revealed that a reduction in the ozone inactivation curve shoulder during pre-treatment resulted in a similar reduction in the shoulder of the secondary inactivation curve. The

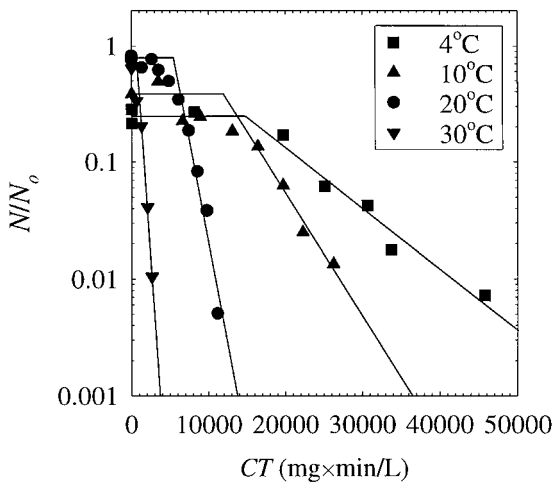

Fig. 5. Effect of temperature on the kinetics of primary inactivation of *C. parvum* oocysts (lots A) with monochloramine at pH 8.

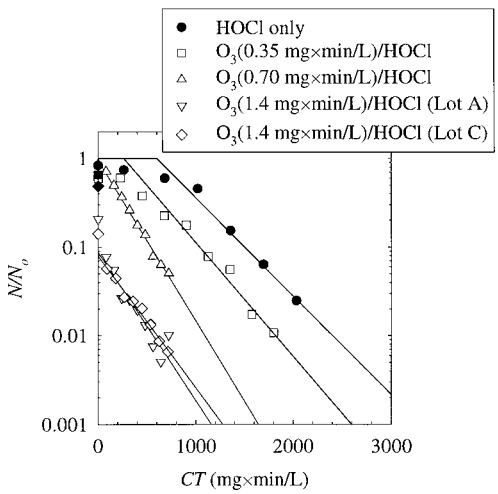

Fig. 6. Effect of ozone pre-treatment CT (20°C, pH 7) on the kinetics of secondary inactivation of *C. parvum* oocysts (lot A unless otherwise indicated) with free chlorine (20°C, pH 6).

pseudo-first-order rate constant for the secondary curve corresponding to the partial reduction of the curve shoulder (ozone pre-treatment CT = 0.35 mg × min/l) was estimated at k = 0.00295 l/(mg × min) by fitting the data with equation (3). This value was 16% higher than that of 0.00255 l/(mg × min) for the post-shoulder primary inactivation with free chlorine. In contrast, k values for the secondary curves for which the entire shoulder was eliminated by ozone pre-treatment (ozone pre-treatment CT = 0.7 and 1.4 mg × min/l) were found to be approximately 1.3–1.6 times that of the primary inactivation with free chlorine. This synergistic effect was consistent with that observed by Driedger *et al.* (2000) for sequential inactivation schemes involving ozone as primary disinfectant and hypochlorous acid as secondary disinfectant. Notice that in agreement with the observation by Driedger *et al.* (2000), an initially faster rate of secondary inactivation for the curves corresponding to ozone pre-treatment CT = 1.4 mg × min/l was observed. As depicted in Fig. 6, the viability measured after ozone pre-treatment deviated from the intercept of the line obtained by linear regression of the subsequent data points.

Experimental results obtained for the ozone/monochloramine experiments also revealed the occurrence of synergy as depicted in Fig. 7. A partial reduction in the inactivation curve lag phase by ozone pre-treatment at CT = 0.35 mg × min/l resulted in partial reduction of the secondary inactivation shoulder. The rate constant for the post-shoulder pseudo-first-order inactivation of the pre-treated oocysts, k = 0.000794 l/(mg × min), was within 1% of that for primary inactivation with monochloramine, k = 0.000801 l/(mg × min). In contrast, the rate of secondary inactivation after removing the shoulder by ozone pre-treatment at

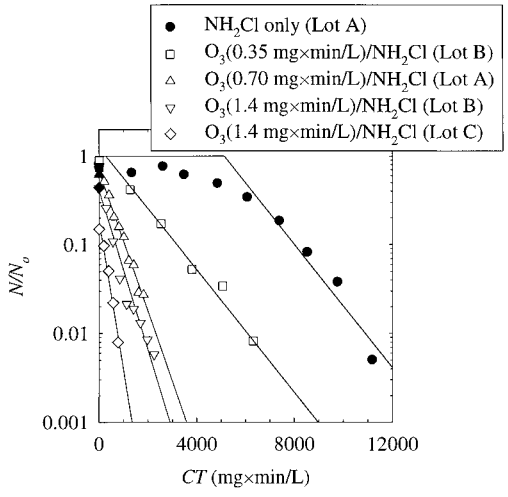

Fig. 7. Effect of ozone pre-treatment CT (20°C, pH 7) on the kinetics of secondary inactivation of *C. parvum* oocysts (lots A, B, C) with monochloramine (20°C, pH 8).

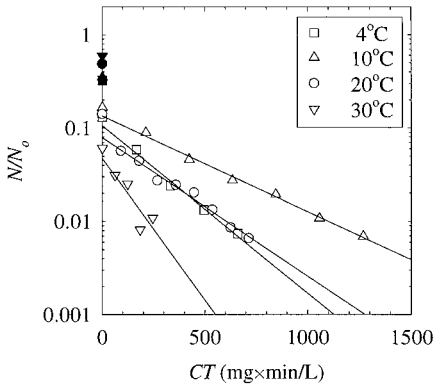

Fig. 8. Effect of temperature on the kinetics of secondary inactivation of *C. parvum* oocysts (lot C) with free chlorine (pH 6) after ozone pre-treatment (pH 7).

CT = 0.7 mg × min/l, k = 0.00182 l/(mg × min), was 2.3 times that for primary inactivation with monochloramine. Additional ozone pre-treatment (CT = 1.4 mg × min/l) resulted in even greater synergy. Secondary inactivation rate constants were k = 0.00208 l/(mg × min) for lot B, and k = 0.00385 l/(mg × min) for lot C, or 2.6 and 4.8 times greater than that for primary inactivation with monochloramine. The different curves obtained with lots B and C, despite receiving the same level of ozone pre-treatment, was likely the result of differences in oocyst resistance to inactivation as supported by comparing the corresponding ozone inactivation curves in Fig. 1.

In addition to the synergy associated with the increase in post-shoulder inactivation rate, an additional synergistic effect also observed was that the more pronounced lag phases of the primary inactivation curves for free chlorine and monochloramine (Figs 4 and 5) were removed by removing the less pronounced shoulder of the ozone inactivation curve (Fig. 1).

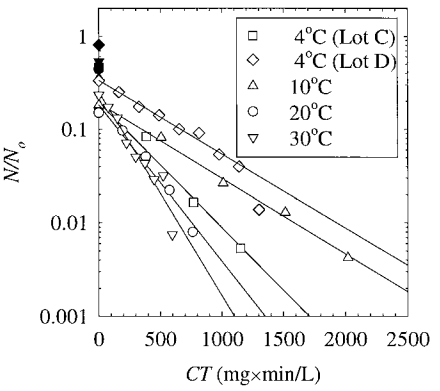

Fig. 9. Effect of temperature on the kinetics of secondary inactivation of *C. parvum* oocysts (lot C unless otherwise indicated) with monochloramine (pH 8) after ozone pre-treatment (pH 7).

Effect of temperature. The role of temperature (4–30°C) in sequential inactivation of *C. parvum* oocysts with ozone/hypochlorous acid and ozone/monochloramine was assessed with oocysts from lot C. Respective experimental results are presented in Figs 8 and 9. Once again, open symbols were used for secondary inactivation curves and filled symbols for the corresponding control samples (without ozone pre-treatment). Target ozone pre-treatment *CT* values selected to reduce oocyst viability to 20% were 10, 3.5, 1.4, and 0.3 mg × min/l for the temperatures of 4, 10, 20, and 30°C, respectively, as indicated with arrows in Fig. 2.

Consistent with experimental results discussed previously for 20°C, ozone pre-treatment in excess of CT_{lag} also resulted in secondary free chlorine inactivation curves without a shoulder at temperatures in the range of 4 to 30°C (Fig. 8). The corresponding secondary rate constants obtained by linear regression of the inactivation curves are plotted according to equation (4) in Fig. 3. The effect of temperature on the rate of secondary inactivation with free chlorine after ozone pre-treatment was consistent with the Arrhenius relationship for temperatures in the range of 10–30°C. Surprisingly, the rate of secondary inactivation at 4°C was faster compared to those at higher temperatures. An explanation for this erratic result will be offered after presenting the results for the ozone/monochloramine tests.

Similar results were observed for oocysts treated sequentially with ozone/monochloramine (Fig. 9). In general, the rate of inactivation decreased with decreasing temperature in the range of 10–30°C according to equation (4) as depicted in Fig. 3. Furthermore, an apparently erratic faster rate was also observed at 4°C. One more interesting observation was that the rate constants for the secondary inactivation of both hypochlorous acid and monochloramine were approximately the same at all temperatures. Fitting of the experimental k values at 10–30°C with equation (4) resulted in $A = 2.84 \times 10^4$ l/(mg × min) and $E_a = 38{,}530$ J/mole for free chlorine, and $A = 8.00 \times 10^2$ l/(mg × min) and $E_a = 30{,}080$ J/mole for monochloramine. The lower apparent activation energies are indicative of changes in the overall mechanism responsible for the inactivation of *C. parvum* oocysts with hypochlorous acid and monochloramine after pre-treatment with ozone. The apparently erratic rate constant values observed at 4°C were approximately 2.7 (hypochlorous acid) and 1.8 (monochloramine) times higher than those predicted with the Arrhenius expression based on the higher temperature data. Interestingly, these deviations are of the same order of magnitude as that discussed previously for the primary inactivation data with ozone at 30°C (rate constant 2.25 times higher than that predicted with the Arrhenius expression based on 4–20°C). A common condition for all of these three tests was that they were the last three tests performed with oocysts from lot C after storage times of 110–115 days. Apparently, a sudden change in oocyst resistance to disinfectants took place between days 103 and 110. This conclusion is consistent with the observation reported by Driedger *et al.* (2000) for primary inactivation with ozone. These authors found that the rate constant for the inactivation of 103-day-old oocysts with ozone at 20°C was approximately twice that reported by Rennecker *et al.* (1999a) for younger oocysts. Furthermore, the conclusion that the secondary inactivation rates obtained at 4°C were erratic was also supported by the results obtained for an additional ozone/monochloramine tests performed with younger oocysts from lot D (Test OM-8, Table 1). The experimental results are presented in Fig. 9, and the corresponding rate constant is plotted in Fig. 3. The experimental rate constant of 0.00182 l/(mg × min) was only 7% higher than that predicted by equation (4) with $A = 8.00 \times 10^2$ l/(mg × min) and $E_a = 30{,}080$ J/mole.

The temperature dependence for primary and secondary inactivation rate constants presented in Fig. 3 indicate that the synergistic effects of sequential disinfection schemes are more pronounced at low temperatures. The ratio of expressions of the form of equation (4) with collision factor and activation energy values given previously for primary and secondary inactivation with hypochlorous acid and monochloramine can be used to estimate the level of synergy at each temperature. For example, the rate of secondary inactivation was 1.1–2.8 times faster than the post-shoulder rate of primary inactivation with hypochlorous acid at respective temperatures of 30–10°C. In the case of monochloramine, the rate enhancement was 2.4–9.2 times again for respective temperatures of 30–10°C. The rate enhancement estimated for monochloramine at

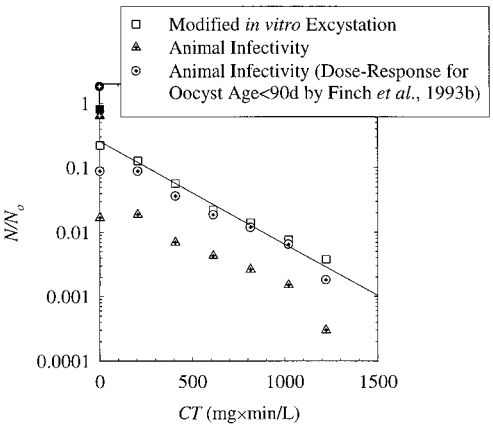

Fig. 10. Comparison of the kinetics of *C. parvum* oocysts (lot D) inactivation with ozone/monochloramine obtained with oocyst viability assessed by modified *in-vitro* excystation and animal infectivity methods.

4°C, verified with oocysts from a different lot, was approximately 14 times. However, additional testing with a single lot of oocysts would be necessary to confirm this enhancement, as well as to extend the study to temperatures near the freezing point. The availability of such information should be most valuable for winter operation of drinking water disinfection systems.

Comparison of modified in-vitro excystation and animal infectivity methods

Results obtained for the experimental set (test OM-9, Table 1) designed to compare viability by modified *in-vitro* excystation and animal infectivity methods are presented in Fig. 10. The *in-vitro* excystation results with oocysts from lot D were consistent with those obtained for lot C. The secondary inactivation rate constant for test OM-9, k = 0.00368 l/(mg × min), was within 10% of the value k = 0.00386 l/(mg × min) obtained for test OM-5 (see Fig. 9).

The animal infectivity results were interpreted with equation (2). However, a_1 and a_2 values reported in the literature for Iowa strain *C. parvum* oocysts vary widely (Finch *et al.*, 1993b; US EPA, 1999; Gyürék *et al.*, 1999). The curve with triangular symbols in Fig. 10 is based on the dose-response parameters $a_1 = 6.752$ and $a_2 = 3.611$ (US EPA, 1999) recommended by the University of Arizona. As depicted in the figure, the inactivation efficiency based on animal infectivity was approximately 0.7-log greater compared to that based on modified *in-vitro* excystation. An effort was made to assess other correlations that would provide lower levels of inactivation efficiencies. For example, parameters obtained by fitting dose-response data reported by Finch *et al.* (1993b) for oocysts younger than 90 days old with equation (2) were $a_1 = 12.46$ and $a_2 = 5.364$. The circular symbols represented in Fig. 10, obtained with these new parameters, matched the modified *in-vitro* excystation results more closely. These comparisons revealed that secondary inactivation data based on the modified *in-vitro* excystation method are generally consistent with animal infectivity results and that any discrepancies observed could be due to analytical variability.

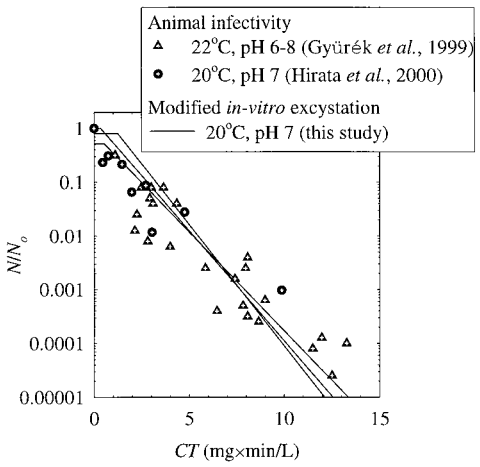

Fig. 11. Comparison of the kinetics of *C. parvum* oocysts inactivation with ozone determined in this study with modified *in-vitro* excystation and animal infectivity data reported by Gyürék *et al.* (1999), and Hirata *et al.* (2000).

Similar levels of consistency between modified *in-vitro* excystation and animal infectivity methods were found for primary inactivation of *C. parvum* oocysts with ozone (Rennecker *et al.*, 1999a) and chlorine dioxide (Ruffell *et al.*, 2000). Additional evidence of consistency between these viability methods for primary inactivation with ozone is presented in Fig. 11. The inactivation curves obtained by fitting the data (pH 7, 20°C) in Fig. 1 with equation (2) were extrapolated to greater inactivation efficiencies. The resulting lines are compared to the data reported by Gyürék *et al.* (1999) for the inactivation of Iowa strain *C. parvum* oocysts with ozone at pH 6–8 and a slightly higher temperature of 22°C. Also shown in the figure are the animal infectivity results reported by Hirata *et al.* (2000) for the inactivation of rodent-source (originally isolated from a human patient) *C. parvum* oocysts at pH 7 and 20°C. As depicted in Fig. 11, good agreement is observed among the three data sets confirming the consistency between viability assessment methods.

CONCLUSIONS

The inactivation kinetics of *C. parvum* oocysts with single disinfectants (ozone, hypochlorous acid, and monochloramine) was characterized by a lag phase with little inactivation followed by a pseudo-first-order decrease in viability. The lag phase was found to be most predominant for monochloramine, followed by hypochlorous acid and then ozone. The respective fractions of the total *CT* required to achieve 99% inactivation of *C. parvum* oocysts that were used to overcome the lag phases were 55, 40 and 20%.

Removal of the less pronounced lag phase by ozone pre-treatment resulted in the absence of the more pronounced lag phase in secondary inactivation with both hypochlorous acid and monochloramine. An additional synergistic effect was an enhancement in the rate of secondary inactivation with both hypochlorous acid and monochloramine after complete removal of the lag phase by ozone pre-treatment. A stronger synergy was observed at lower temperature. The rate of secondary inactivation was 1.1–2.8 (hypochlorous acid) and 2.4–9.2 (monochloramine) times faster than the corresponding post lag-phase rate of primary inactivation at respective temperatures of 30–10°C.

Inactivation efficiencies obtained by the modified

in-vitro excystation method were found or shown to be consistent with animal infectivity data for both primary inactivation with ozone and secondary inactivation with monochloramine after ozone pretreatment.

The results presented in this manuscript indicate that drinking water utilities using ozone as primary disinfectant and having sufficient contact time with the secondary disinfectant in product water clear wells or reservoirs can provide substantially greater protection against *C. parvum* oocysts compared to the effect of the primary disinfectant alone. Furthermore, this information can also serve as a guideline for the design of new sequential disinfection systems using ozone/monochloramine and ozone/free chlorine.

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